

BIOACTIVE GLASSES MODULATE TISSUE-SPECIFIC GENE EXPRESSION IN RAT NASAL CHONDROCYTE CULTURES*A Asselin, A Berdal, J-M Sautier¹*¹ *Laboratoire de Biologie Orofaciale et Pathologie, INSERM E 0110, Université de Paris VII, UFR Odontologie, Paris, France*

INTRODUCTION: The replacement of bone tissue is a common problem in medicine and dentistry. Autografts are known to be the ideal bone replacement solution for cases of bone loss in both orthopedic and periodontal surgery. However, problems such as limited donor supply have led us to think about other types of bone substitutes such as alloplastic materials. One category of these biomaterials is the bioactive glasses tested in this study. Previous studies have shown that this material promotes cellular differentiation and can be entirely integrated to bone by forming a chemical bond with the host tissue. However most of these studies are made on bone cells, for instance the studies realized in our laboratory^{1, 2}, have demonstrated *in vitro* a stimulation of osteogenesis. It is of interest that during long bones formation, the process is preceded by a stage of endochondral ossification, in other words the formation of a cartilaginous tissue that is resorbed and replaced later by bony tissue. This process also occurred during bone healing following trauma or placement of implants. For this reason we decided to evaluate the effect of 45S5[®] bioactive glasses on the behavior of chondrocytes *in vitro*. To achieve our goal we used primary cell culture, that mimics in the closest way, what happens during endochondral ossification *in vivo*, from the stage of cell proliferation till the stage of hypertrophic chondrocyte and finishing by matrix mineralization³. We analyzed the phenotypic expression of different cartilaginous markers such as type II collagen, aggrecan, Cbfa1, Sox 9, Indian Hedgehog (Ihh), alkaline phosphatase and type X collagen.

METHODS *Biomaterial tested* : the biomaterials used were 45S5[®] bioglasses composed of (in weight %) : 45 % SiO₂, 24,5 % CaO, 24,5 Na₂O, and 6% P₂O₅. 60S granules were used as control in our experiments.

Culture model : chondrocyte were enzymatically isolated from nasal septum of 21 day- old fetal Sprague Dawley rats, as previously described³. Briefly, nasal septum were aseptically dissected and fragments incubated in phosphate buffered solution with collagenase (400 U/ ml) and hyaluronidase (750 U/ ml), for 2 hr at 37°C. Then cells were dissociated from the cartilage fragments, and plated

at 3.5×10^4 cell/cm². The glass granules were added on day 1 of culture. All cultures were incubated in DMEM supplemented with 10% foetal calf serum, β -glycerophosphate, and ascorbic acid, in a humidified atmosphere of 5% CO₂ in air at 37°C.

Transmission electron microscopy : The cells cultured with bioglass particles, were fixed on day 10 in Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde) for 1 hour. After several rinses in 0.2M sodium cacodylate buffer (pH 7.4), cell cultures were post-fixed for 1 hour in osmium tetroxide diluted in 0.2M sodium cacodylate buffer. The cells were then dehydrated in graded series of ethanol and left overnight in a mixture of absolute ethanol and epon (1:1). The next day the cells were embedded in Epon Araldite and incubated at 60°C for 1 day. Ultrathin sections were performed, collected on copper grids, and stained with 2.5% uranyl acetate in absolute ethanol for 4 minutes and lead citrate for 2 minutes. The sections were then examined under a TEM (Philips CM-12).

Alkaline Phosphatase activity : The specific activity of alkaline phosphatase was assayed in cell layers as released p-nitrophenol from p-nitrophenolphosphate. The optical density was read at 410 nm, and the enzyme activity was expressed as unit per milligram of total proteins (estimation of protein content was carried out using the Pierce BCA protein assay kit).

Total RNA isolation and real-time RT-PCR : total RNA was extracted at day 5, 9, 12, 16, and 20 using a phenol/chloroform method (Tri reagent[®]). The concentration and purity of RNA were determined by light absorbance at 260nm and by calculating A₂₆₀/A₂₈₀ ratio respectively. The integrity was confirmed by electrophoresis on an agarose ethidium bromide gel. Two micrograms of total RNA from each sample was used for Reverse Transcriptase (superscript II[®]), using oligo(dT) primer. Then cDNA obtained were used for quantitative PCR (light cycler, Roche diagnostic[®]) using forward and reverse primers with sybrgreen method. Each RNA sample was analyzed in triplicate for Aggrecan, Sox9, Type II and X collagen, Cbfa1/Osf2 and Ihh mRNA, and was corrected for GAPDH mRNA levels, used as a reference.

RESULTS: Phase contrast microscopy showed multiple zones of mineralization, but more abundant in cultures with 45S5 when compared to 60S. Transmission electron microscopy showed the establishment of an intimate contact between the cells and the granules, a collagen-rich matrix was also observed in both cultures. Alkaline phosphatase activity measures at day 5, 8, 12, 15 and 18, showed that the enzyme gradually increased up to day 15 and then decreased on day 18. The levels of ALP activity were higher at all times, in cultures with 45S5 bioglasses when compared to control. Furthermore analysis of the expression of specific chondrogenic markers was determined at day 5, 9, 12, 16, and 20 using real-time RT-PCR. Chondrocytes in both cultures expressed the specific markers but at a higher level in 45S5 cultures when compared to control. Finally, the tissue-specific gene expression coincided with the temporo-spatial pattern observed during *vivo* chondrogenesis.

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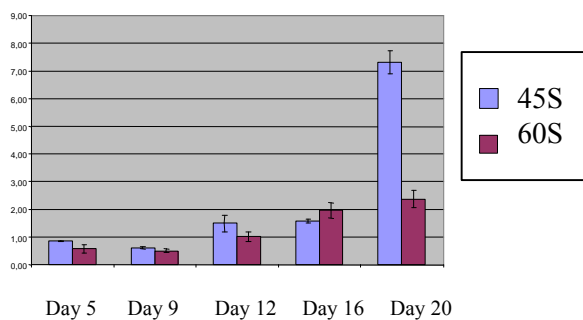


Fig. 1 Effect of 45S5 granules on type X collagen expression, in primary chondrocyte culture.

DISCUSSION & CONCLUSIONS: Our results have shown that bioactive glasses cannot only support the proliferation of chondrocytes *in vitro*, but can also stimulate their differentiation. Alkaline phosphatase activity, increased in cultures of 45S5. The stimulation of the expression of various early and late chondrogenic markers, as well as transcriptional factors such as Sox 9 or Cbfa-1, indicates that bioactive glasses can modulate the expression of these genes, and promotes cellular differentiation. For these reasons, bioactive glasses appeared to be an ideal scaffold that supports and promotes cell proliferation and differentiation in the domain of tissue engineering.

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